Nutritional 'Omics' Technologies for Elucidating the Role(s) of Bioactive Food Components in Colon Cancer Prevention

New Nutrition, Proteomics, and How Both Can Enhance Studies in Cancer Prevention and Therapy[1]

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ABSTRACT The increased application of MS technologies to nutrition and cancer prevention research has enabled unique insights into the health benefits of polyphenols. Polyphenols are phytochemicals that appear to have chemical properties that provide valuable health benefits when ingested. In particular, experiments have suggested that grape seed proanthocyanidins, oligomers of the catechin family of polyphenols, may have health benefits, possibly due to their capacity to be oxidized. Two-dimensional gel proteomics technology identified specific rat brain proteins that were differentially affected after ingestion of grape seed extract. Beneficial changes in the expression of these proteins were observed relative to changes seen in the brains of Alzheimer disease patients at autopsy and of transgenic mouse models of dementia. These findings were consistent with the hypothesis that grape seed polyphenols may have neuroprotective activity. Previous experiments showed that grape seed extract was significantly chemopreventive in a rat model of breast cancer, but this depended on the specific diet in which the grape seed was administered. Thus, phytochemicals such as polyphenols may have health benefits in mammalian tissues unrelated to classical nutritional deficiency models. This report illustrates how experimental approaches that combine proteomics technologies with a dietary intervention with specific phytochemicals in normal animals can enhance studies on cancer prevention and treatment. J. Nutr. 135: 2715–2718, 2005.

KEY WORDS: • *2D gels* • *chemoprevention* • *nutriproteomics* • *diet and cancer* • *chemotherapy*

The concept that the consumption of foods, or food patterns, can prevent both deficiency diseases and prevent chronic diseases dates back to antiquity. Current nutrition research is using sophisticated technologies to identify the molecular basis for the activity of various dietary chemicals. For example, these techniques may permit the identification of proteins whose expression are affected by such compounds in normal tissues and allow for the identification of proteins that mediate normal cellular functions supported by phytochemicals. As such, these hitherto unidentified proteins may be potential players in the disease process that may develop in the absence of the phytochemicals. In this report we discuss various aspects of two-dimensional (2D)[3] gel proteomics technology that contributed to the identification of specific proteins in rat brain that were affected differentially by dietary administration of grape seed extract (GSE). GSE is a commonly available dietary supplement, to which anti-oxidant properties were first described by Masquelier et al. (1). Previous experiments that suggested that the protective effects of GSE against carcinogen-induced rat mammary cancer might be diet context specific have generated valuable tissues for proteomics analysis. The concept that the physical patterns of protein spots resolved on 2D gels themselves is a tool with which cancer and its prevention or treatment can be studied, irrespective of the identities of the specific proteins within the spots, is presented as an analytical end in itself.

MATERIALS AND METHODS

Generating brains for proteomics analysis of actions of GSE in normal rat brain. Normal weanling female Sprague Dawley rats (Charles River), were fed an AIN-76A diet (Teklad Industries) (2)

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[3] Abbreviations used: 2D, two-dimensional; DMBA, dimethylbenz[a]anthracene; GSE, grape seed extract; IEF, isoelectric focusing; IPG, immobilized pH gradient; LC-MS/MS, liquid chromatography tandem MS; MOWSE, molecular weight search engine; Qtof2 MS, quadrupole orthogonal time of flight MS.

upon receipt. After 2 wk, they were segregated into 2 dietary groups, one group ($n = 5$) received the AIN-76A diet supplemented with 5% GSE (Kikkoman), and a second group ($n = 5$) received unsupplemented AIN-76A diet. Six weeks later, the animals were euthanized by carbon dioxide asphyxiation, and the whole brain above the brain stem was dissected out from each rat, snap frozen in liquid N_2, and stored at −80°C until analysis. These animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

2D gel electrophoretic analysis of rat brain homogenates. Rat brain samples were processed for 2D electrophoresis as described (3); a sagittal section was homogenized in isoelectric focusing (IEF) lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L DTT) [adjusted with 1 tablet of EDTA-free complete protease inhibitor (Roche Diagnostics) within 30 min of use] at room temperature, clarified by centrifugation at 100,000 × *g* for 30 min at 22°C, the supernatant aspirated, and a protein concentration determined for the latter using the BioRad Bradford reagent (BioRad Laboratories). Triplicate immobilized pH gradient (IPG) strips (GE Healthcare) containing 4–7 pH gradients were rehydrated overnight over IEF lysis buffer containing 200 μg total brain protein per strip under mineral oil. IEF was carried out the next day following manufacturer's recommended protocol for 11 cm IPG strips, using the IPGphor apparatus (GE Healthcare). After the IEF, strips were drained on filter paper of excess mineral oil, frozen overnight at −80°C, then equilibrated for SDS-PAGE, laid on top of 10–20% gradient acrylamide gels (Criterion gels; BioRad Laboratories) and electrophoresed until the tracking dye reached the bottom of the gel. The gels were subsequently stained with colloidal Coomassie Brilliant Blue (Pierce Biotechnology), and their images acquired with a BioRad GS 710-Calibrated Imaging Densitometer. Shown in **Figure 1** is how the animal experiment generated the 2D gel images. Image analysis of the 30 gel dataset was accomplished with PDQuest image analysis software (BioRad Laboratories). The *x*,*y* coordinates and peak intensities for each gel spot were downloaded and subjected to statistical analysis using Statistical Analysis Systems (SAS Version 09, SAS Institute) (4), which ultimately determined those spots whose normalized intensities were significantly different between the GSE group vs. the control group. Standard *t* test and *f* test analysis identified spots that differed in intensity and in variability of intensity, respectively. The statistical algorithms used to analyze these data are described elsewhere (5).

Putative protein identification by peptide mass fingerprint analysis. The significantly different gel spots were excised, subjected to ingel digestion with trypsin, and subjected to peptide mass fingerprint analysis via matrix-assisted laser desorption ionization time of flight MS according to established procedures (http://donatello.ucsf.edu/ingel.html) (3,6). A Voyager DePro mass spectrometer (Applied Biosystems) was used and internally calibrated with trypsin autolysis peaks and externally calibrated with angiotensin, bradykinin, and neurotensin. The MASCOT search engine at http://matrixscience-.com was used to search the protein sequence database. Such procedures yielded the putative identities listed in **Table 1**, along with the MOWSE (molecular weight search engine) scores and the gene accession numbers.

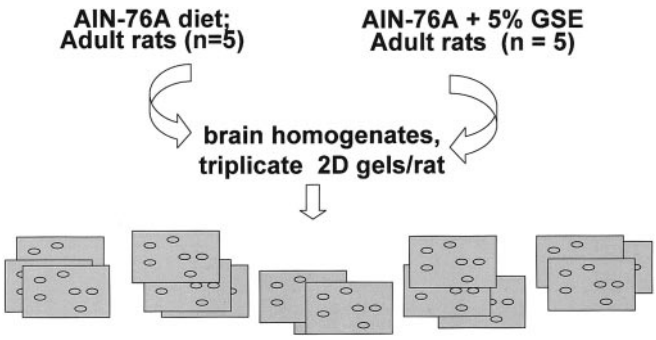

FIGURE 1 The animal experiment that generated brain samples for proteomics analysis. Sprague Dawley female rats (35 d old, $n = 5$) received either AIN-76A or supplemented with 5% GSE. Animals were euthanized at 6 wk, proteins in the whole-brain homogenates were resolved on replicate 2D gels (shown schematically at the bottom), and analyzed by image and statistical analysis to determine those gel spots that were significantly different between the 2 dietary groups due to the GSE treatment. These spots were excised and processed for MS analysis to identify the polypeptides.

Confirmation of protein identities by sequence analysis by liquid chromatography-tandem MS. Putative identifications were confirmed by liquid chromatography tandem MS (LC-MS/MS) on a hybrid quadrupole orthogonal time of flight MS (Qtof2 MS) (Waters), as described previously (3). LC-MS/MS involved chromatographic resolution of the peptides on a Biowide C_{18} 3 μm 300 A° column (Supelco), collision-induced dissociation and electrospray of the eluate onto a Qtof2 MS. A full description of the proteomic analytical methods used to generate the data described here are given in Deshane et al. (3).

Chemoprevention studies. Female Sprague Dawley rats were segregated into dietary groups and given either 0%, 1.25%, or 5% GSE-supplemented AIN-76A diet starting at 35 d of age (20 per group). At 50 d, all were given dimethylbenz[a]nthracene (DMBA) (80 mg/kg b.w.) by gavage in sesame oil. Two control groups received AIN-76A alone, and AIN-76A and GSE, but no DMBA. Animals were monitored daily for overall health and were palpated and counted for tumors weekly. Animals were euthanized 6 mo after DMBA administration, and the final body weights and the tumor counts were made. In the second round of analysis, the entire experiment was repeated as above with the same doses of GSE, except 4% rodent diet (Teklad Industries) was used instead of AIN-76A diet. A complete description of the procedures used in the chemoprevention studies have been previously described (7).

RESULTS AND DISCUSSION

A typical pair of 2D gel images in the 30 gel image data set is shown in **Figure 2**. These 30 were treated as one data set to image analysis using PDQuest software. Statistical analysis of the numeric data from the image analysis, independent of the 2D gel images, determined spots that were significantly different in intensity between the 2 dietary groups (circled), and those that were significantly different in variability of intensity between the 2 dietary groups (boxed). The complexity of the mammalian brain proteome is evident even at 200 μg protein loading. Approximately 200 spots were determined to be common among all gels in either group. Given that the human genome contains ~20,000 genes and therefore the proteome in any given tissue can be predicted to be at least 20,000 polypeptides, the 200 gel spots analyzed in this study represented at most 1% of the proteome. Finally, while specific and quantitative differences in protein spots were determined, the overall similarity in pattern of protein spots for the rat brain proteome was evident from gel to gel, indicating the reproducibility inherent in the technique.

What is obtained routinely in proteomics experiments is shown in Table 1. In particular, the gene accession number is a valuable entity for that protein/gene product; it can be entered on any one of a number of Web sites or links at Web sites, such as www.ExPaSy.org, to discover sequence or functional information about the protein. All data ultimately contribute to a deeper understanding of what the change measured for that protein in one experiment might mean physiologically.

In this study, a small number of proteins was determined by statistical analysis to be significantly different in intensity or in variability between the gels representing the 2 dietary groups. Informatics analysis of these revealed that instead of multiple random or nonrelated functional categories, these proteins belonged to 3 functional categories, heat-shock protein, en-

TABLE 1

Proteomics of actions of GSE in rat brain

Protein identity[1]	Functional category[2]	MOWSE score[3,4]	Gene accession number	LC-MS/MS[5]
Heat shock protein 60	Chaperone	1.26E+045	P19227	Yes
Heat shock protein 70	Chaperone	110	gi|4103877	Yes
Heat shock protein 71	Chaperone	105	gi|123644	Yes
Creatine kinase brain beta chain	Energy (generation)	1.66E+05	P07335	Yes
α and γ enolases	Energy (utilization)	6.6E+05	P04764	Yes
		95	P07323	
Glial Fibrillary Acidic Protein	Cytoskeleton	88	P47819	Yes
Vimentin	Cytoskeleton	93	gi|202368	Yes
Actin	Cytoskeleton	96	gi|113307	Yes

[1] The putative identification obtained via database search via MASCOT search engine, at http://www.matrixscience.com.
[2] Determined by informatics search at http://www.ExPaSy.org.
[3] The molecular weight search engine score generated for each putative identification at MASCOT. Scores above 71–75% are considered valid matches.
[4] The scores expressed were generated within PS1 software (Applied Biosystems, Foster City, CA). Scores above E+04 are considered significant.
[5] LC-MS/MS was carried out for a number of the identifications generated by peptide mass fingerprint analysis, for confirmation.

ergy generation/regulation, and cytoskeleton (Table 1). None of the fold changes measured for the proteins was greater than 2-fold (3). It should be kept in mind, however, that these data were obtained for a single time point. Thus, while rigorous, these data are a starting point in the discovery process that proteomics enables.

One final point that needs reckoning is that the measured fold change for an effect, even if beneficial, such as increased expression of a specific protein, might be small. The biological basis is that the cell wants to maintain homeostasis and thus minimizes any change. Hatzimanikatis and Lee (8) showed that in response to a stimulus, one mRNA increased and stayed elevated and its corresponding protein levels increased. Another protein, however, increased along with its mRNA initially, but then its level was dramatically reduced shortly thereafter (8), as if the different level of this protein was not desired by the cell, and was dealt with by being turned over. Normal intracellular dynamics involves interactions among different structural and enzymatic activities. A mere 2-fold change in the amount of a protein could have nontrivial consequences in cell morphology or motility if that protein were an enzyme that affected the stability of the cytoskeleton, for example.

Proteomic signatures of the effect of GSE on rat brain may provide insights into its neuroprotective and chemopreventive effects. In other words, in the molecular pathways the proteins involved represent may provide unique insights into a particular cancer-related phenotype (e.g., angiogenesis, activation of protein tyrosine kinases, etc.) impacted by a specific polyphenol. Ultimately, such information will be the basis for the development of preventives or therapeutic reagents.

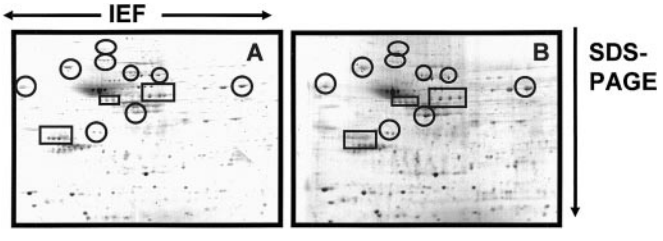

FIGURE 2 Representative images of the 2D gels of the rat brain homogenates. These images of 2 real gels show the patterns of protein spots resolved by the orthogonal separation modes, isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second. The image analysis via PDQuest and subsequent statistical analysis determined that the spots indicated by the circles and rectangles, respectively, differed between the 2 groups of gels in their intensities, and in their variances, respectively.

While an important goal of this research is to understand the role of polyphenols in carcinogenesis, the pharmaceutical industry will be more interested in identifying effective preventive or therapeutic agents using high throughput assays without necessarily needing to understand the mechanisms or what proteins they affect. Once candidate compounds are identified; however, knowing what are the target proteins or pathways is invaluable in developing compounds with higher specificity or efficacy. Thus, both basic cancer research and targeted chemopreventive or chemotherapeutic compound discovery can ultimately involve searching for "significant" proteins. This search can be greatly enhanced by proteomics approaches that identify changes in patterns of protein expression and modifications in living systems in response to specific stimuli. Aside from the high throughput nature of the technology, proteomics allows rigorous analysis of changes in patterns of protein expression without an a priori prediction of which proteins are involved.

An illustrative example of the application of GSE to breast cancer prevention has been recently demonstrated (7). Based on previous demonstrations in cell culture experiments that grape and tea polyphenols have anticancer properties (9–14), we hypothesized that GSE might inhibit carcinogen-induced breast cancer in rats. Dietary supplementation with 5% GSE inhibited the incidence of DMBA-induced mammary tumors in Sprague-Dawley rats; an unexpected result was that this effect of GSE was not seen when the GSE was administered in a chemically defined rodent diet, AIN-76A (7). Chemopreventive efficacy of the GSE was seen when it was administered in 4% rodent diet, a diet based on plant proteins (soy, barley, and wheat) vs. milk proteins in AIN-76A (both diets from Teklad Industries). Earlier studies showed that administering GSE to rats did not inhibit breast cancer but did inhibit colon cancer (15); whether the differences between our results and those previously reported were due to dose or dietary differences is not known at present. Our results (7) suggest that the composition of the diet in which a purported chemopreventive compound is administered can determine whether the bioactivity of the compound being tested is expressed.

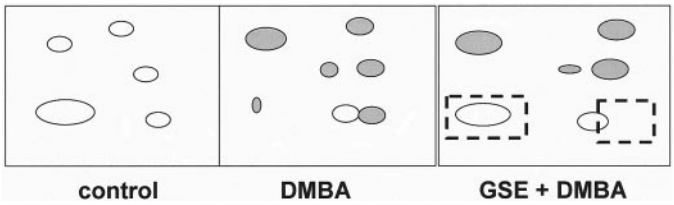

FIGURE 3 How 2D gel patterns can enhance studies of cancer biology/prevention. 2D patterns of gel spots are unique for a given sample and for a given set of 2D gel electrophoretic conditions (gel labeled "control"). Therefore, changes in these patterns, determined by software-assisted analysis, where gel images are digitally overlaid, result in a subpattern of spots that are different in response to a treatment such as DMBA (gel labeled "DMBA," where the shaded spots are different in intensity from their counterparts in the "control" gel). The final 2D gel pattern, of mammary tissue from an animal given GSE prior to DMBA reveals yet another subpattern, of differences induced by the DMBA that were prevented by the GSE (gel labeled DMBA + GSE) (indicated by the dashed rectangles). These would be strong candidates for proteins implicated in the chemopreventive actions of GSE against DMBA-induced mammary cancer.

The molecular basis for the differences in efficacy of the GSE against the DMBA-induced mammary tumors between the 2 diets is not known. Thus, the mammary tissues from the animals in these studies are prime candidates for analysis by 2D gel or other proteomics methods in combination with MS. A simple scenario for analysis is shown in **Figure 3**. An altered pattern of protein expression induced by the DMBA carcinogen in mammary tissues can be determined, then compared with how this pattern differs in mammary tissues from animals that began ingesting GSE prior to the DMBA treatment that were subsequently protected against DMBA-induced mammary tumors. It is important to understand that until the very last set of images, the identities of the proteins in the spots do not need to be obtained for the image analysis data to be meaningful.

This report was not meant as a comprehensive review of proteomics, cancer research, or Alzheimer's disease research. Instead, the goal was to document the health benefits, including chemopreventive activity, of GSE in different rat organs, and to demonstrate how the same proteomics technology that identified and rigorously characterized the proteins affected by GSE in brain could be extended to the study of tissues from cancer studies. Last, but not least, an objective was to emphasize that the "new" nutrition includes diet or dietary components protecting against diseases such as cancers, with effects at a time point distal to the time of ingestion of the dietary component.

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